RESPONSES OF *ASPERGILLUS CARBONARIUS* TO TWEEN 80. MYCELIAL GROWTH, PROTEIN, RNA AND CHITIN CONTENT.

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*This paper is dedicated to my Professor Dr S. Marakis

ABSTRACT : Studies were carried out to investigate the effects of the surfactant Tween 80 on spore germination, mycelial growth and form of *Aspergillus carbonarius* and its protein, RNA and chitin content. The presence of increasing (0.2-1.1%) Tween 80 concentrations in the medium: a) reduced the spore germination time from 24h (in the control) to 3h, b) reduced hyphal diameter and chitin content while hyphal branching frequency was increased resulting in the formation of soft, loose and large pellets and c) increased the mycelium dry weight while its protein and RNA content (%) was reduced.

KEY WORDS: Tween 80, germination, hyphal growth unit (G), *Aspergillus carbonarius*

RÉSUMÉ: Des études, concernant les effets du Tween 80 sur la germination des spores, la forme et la croissance du mycélium d'*Aspergillus carbonarius,* ainsi que sur son contenu en proteines, RNA et chitine, ont été enreprises. Des concentrations croissantes de Tween 80 ont pour conséquences : a) une réduction du temps de germination des spores de 24h à 3h ; b) une diminution du diamètre des hyphes et du contenu du mycélium en chitine, ainsi qu'une augmentation de la fréquence des ramifications ayant pour conséquence la formation de « pellets » lâches, de grande taille ; c) l'augmentation du poids sec de mycélium et une diminution du contenu (en %) du mycélium en protéines et en RNA.

MOTS CLEFS: Tween 80, germination, unité de croissance hyphale (G), *Aspergillus carbonarius*.

INTRODUCTION

Several organic compounds [aliphatic acids (mainly unsaturated), alcohols, aldehydes and surfactants] have been found to affect growth, mycelial form and enzyme production (amylase, cellulase, sucrase, ligninase etc.) of many fungi (Takahashi *et al.*, 1960; Fries, 1973; Rao & Rao, 1975; Panda *et al.*, 1987; Asther *et al.*, 1987; Marakis, 1988; Gomez-Alarcon *et al.*, 1989).

Surfactants, in general, such as Tween 20, Tween 80 etc., modify protein-lipid interactions, solubilize specific proteins and lipids from membranes, lower the surface tension of liquids and alter membrane permeability (Helenius & Simons, 1974).

Since sorbitan polyoxyethylene monooleate (Tween 80) is of great importance in various fungal biotechnological processes because it can stimulate and increase the production and activity of several enzymes (Asther *et al.*, 1987; Gomez-Alarcon *et al.*, 1989) or increase alcohol volumetric productivity in ■ continuous alcohol fermentation (Torrico & Acevedo, 1988) and as a basic constituent of detergents thrown in the environment (seas, rivers etc.) might affect fungal growth, a study of Tween 80 effects on fungal growth physiology is needed.

Important parameters of fungal growth are: a) the spore germination time, since it determines the lag phase time, b) the morphological form of the mycelium because from changes in the morphology, a whole range of biochemical changes and alterations of culture physical properties take place and c) the protein content, since the produced biomass could be used as animal feed (Marakis, 1988).

To the best of our knowledge there is very little information concerning the effects of Tween 80 on fungal growth physiology. Marakis (1988) observed an increase in mycelium dry weight and total mycelial protein as well as the uptake rate of the carbon and nitrogen sources when Tween 80 (2.5 g/litre) was added in culture media of *Rhizopus nigricans* and *Penicillium frequentans*. So far, studies reported by others focus to Tween 80 effects on enzymic productions or activities. Asther *et al.* (1987) showed ligninase production by *Phanerochaete chrysosporium* INA-12 when Tween 80 was supplemented to the culture medium. Gomez-Alarcon *et al.* (1989) showed ■ beneficial effect of Tween 80 in increasing yields of various extracellular enzymes of *Pycnoporus cinnabarinus*.

The aim of this study was to investigate some biochemical and morphological changes induced in *Aspergillus carbonarius* growing under high Tween 80 concentrations.

MATERIALS AND METHODS

Microorganism:

A strain of *Aspergillus carbonarius* (Bainier) Thom isolated from carob bean storehouse soil (Gaitis, 1995) was used.

	Media g l^{-1}					
Components*	M	T	T_1	T_2	T_3	T_4
Sucrose	20	—	20	20	20	20
Tween 80	—	10	2	5	8	11

* All media contained (g l^{-1}): $(NH_4)_2SO_4$: 5; KCl: 0.5; $MgSO_4.7H_2O$: 0.5; K_2HPO_4: 1; $FeSO_4.7H_2O$: 0.01.

Table 1. Culture media (g l^{-1}) used for cultivation of *A. carbonarius*.

Culture media:

For the batch cultures six media were used (Table 1). The pH was adjusted at 5.0-5.5. All culture media were sterilized by autoclaving (15 min, 121°C).

Inoculum preparation:

Peripheral growth zone spores from Czapek-Dox agar cultures were obtained after 5 days of incubation at 30°C. Spores were suspended in quater-strength Ringer's solution for 1 min in a Waring blender.

Batch cultivation:

A. carbonarius was grown in three 100 ml Erlenmeyer flasks containing 30 ml of medium. These flasks were inoculated with $3x10^3$ spores ml^{-1} of medium and incubated at 30°C for 7 days on a rotary shaker (160 rpm).

Spore germination:

Spores were considered to be germinated when their germ tube lengths were one-half of the spore diameter.

Harvesting and drying of biomass:

For the protein, chitin and RNA determination the mycelial mats were harvested by filtration through Whatman No 1 filter paper. Then they were thoroughly washed twice with 25 ml distilled water and freese-dried to a constant weight.

Microscopic observations:

Measurements of hyphal diameter and branching frequency: For microscopic observations, pellets from the cultures were filtered, washed and resuspended for 5 min at 20°C in 0.5N NaOH to separate hyphal clumps. Then the separated hyphae of pellets were placed on slides and measurements were made using a calibrated eyepiece in a Zeiss light microscope (Katz *et al.*, 1972; Morrison & Righelato, 1974).

The hyphal growth unit (G) was determined by the procedure of Trinci (1974):

$$G = \frac{\text{Total length of mycelium } (\mu m)}{\text{Number of tips}}$$

Analytical methods:

— Mycelium chitin was determined according to Plassard *et al.* (1982) method based on the determination of glycosamine by HNO_2 transforming it into 2,5-anhydromannose; this compound reacts with the 3-methyl-2-benzothiazolone hydrazone

hydrochloride (MBTH) producing, in the presence of ferric chloride solution an intense blue colour best absorbed at 653nm.

— The RNA concentration was determined by measuring the absorbance at 260nm of a previously $HClO_4$ extracted and KOH digested mycelium (Benthin *et al.*, 1991).

— Protein content was estimated by the method of Gorsuch & Norton (1969).

Statistical analysis:

The data were calculated as the arithmetic mean of 10 replications ± standard error of the mean.

RESULTS AND DISCUSSION

Tween 80 effects on the spore germination time, hyphal diameter, hyphal growth unit and morphology of *Aspergillus carbonarius* are shown in Table 2.

No mycelial growth in medium T, which contained Tween 80 as sole carbon source, was observed. This means that *A. carbonarius* cannot utilize Tween 80 as carbon source. Our data are in agreement with those of Marakis (1988) and Fries (1973), who reported that the presence of small amounts of aliphatic fatty acids, aldehydes etc., promoting mycelium growth, could not act as nutrients.

Spore germination:

Spore germination time in media containing Tween 80 was 8 times shorter compared to that in medium M without this surfactant. This promotion is probably due to the ability of Tween 80 to promote both uptake and exit of compounds from the cell through modification of plasma membrane permeability (Reese & Maguire, 1969; Marakis, 1988).

Media	Spore germination time (h)	Length of G (μm)	Hyphal diameter (μm)	Mycelium form (mm)
M	24.00	112.7 ± 1.3	6.6 ± 0.1	pellets=2.10 ± 0.26
T_1	3.00	88.0 ± 1.8	3.4 ± 0.1	pellets=2.31 ± 0.14
T_2	3.00	87.4 ± 1.8	3.4 ± 0.1	pellets=2.50 ± 0.12
T_3	3.00	63.3 ± 1.7	2.8 ± 0.2	pellets=2.70 ± 0.15
T_4	3.00	64.0 ± 1.2	2.8 ± 0.1	loose-large floculent masses

Table 2. Effects of Tween 80 on spore germination time, hyphal growth unit (G), hyphal diameter and macroscopic morphology of mycelium (pelletal, filamentous) of *A. carbonarius* cultured in synthetic liquid media with or without Tween 80.

Mycelial growth:

Mycelium dry weight was increased by 5-20% (depending on Tween 80 concentration) compared to the dry weight of medium M (Table 3). Biomass increase might happen because of the alteration of the cell wall permeability resulting in a better nutrient inwards defusion.

The percentage of protein and RNA content was reduced by the presence of Tween 80 as compared to medium M (Table 3). The reduction of the percentage of protein and RNA content cannot be explained as a block of the proteinsynthetic ability of *A. carbonarius*, but it is most likely that proteins are reduced through solubilization by Tween 80 treatment (Helenius & Simons, 1974). However, this reduction might be due to the distribution of the same amount of proteins and RNA into greater amounts of produced mycelial biomass.

Mycelium morphology:

Hyphal growth unit (G), hyphal diameter, pellet character and chitin content of *A. carbonarius* were affected by Tween 80 (Tables 2, 3). Thus:

a) The G length measured on young mycelia early in the growth phase and in hyphae in the center and periphery of the pellets after 7 days of incubation, were found to be almost unchanged. The length of G ranged between 64.0 to 112.7 µm. A reduction of G length in media T_1-T_4, with increasing Tween 80 concentrations, was observed. Marakis (1988) found higher G lengths of *P. frequentans* and *R. nigricans* in the presence of Tween 80. Thus, the G length should depend on fungal strain and culture conditions.

b) The hyphal diameter was reduced by the presence of increasing concentrations of Tween 80 (3.4-2.8 µm) as compared to that of medium M (6.6 µm). This hyphal thinning can be attributed to the reduction of the chitin content, since the cell wall of *A. carbonarius* belongs to the chitin-glucan group (Bartnicki-Garcia, 1968). In the presence of Tween 80 the hyphal chitin content was lower (4-72%) compared to that determined in hyphae grown in medium M. Chitin content reduction is possibly due to Tween 80 effect on chitin synthetase synthesis or activity.

Media	Dry weight (mg)	Protein content % on dry weight	Chitin content mg g^{-1} of mycelium	RNA (%)
M	190.0 ± 4.8	33.8	290.0	2.6
T_1	200.0 ± 2.4	25.0	278.5	1.6
T_2	208.0 ± 23.9	24.4	271.4	1.7
T_3	212.8 ± 4.2	24.1	214.3	1.9
T_4	229.5 ± 5.7	24.0	169.0	1.9

Table 3. Tween 80 effects on mycelium dry weight, protein, RNA and chitin contents of *A. carbonarius* cultured in synthetic liquid media with or without Tween 80.

c) The mycelium in medium M formed dense compact regular pellets (Fig. 1A), whereas under the presence of Tween 80 (media T_1-T_4) pellets were soft, irregularly shaped and aggregated into loose large floculent masses as shown in Fig. 1B. It is noticeable that

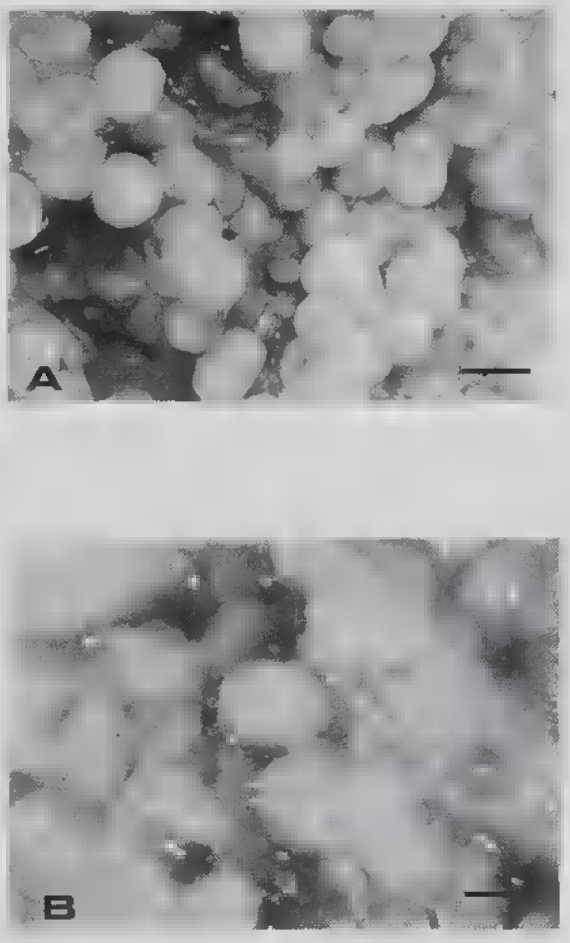

Fig. 1 — Tween 80 effects on *Aspergillus carbonarius*. A. Dense, compact, regular pellets produced in medium M. B. Soft, loose, irregularly shaped pellets produced in medium T_3. Bar = 2mm.

autolytic process was not observed in the pellet center and consequently no proteins were determined in the culture filtrate.

The reduction of the hyphal diameter resulted in the breakage of hyphae which does not favor the formation of dense compact pellets. Our results, concerning the pellet formation, are not in agreement with those of Righelato (1975) and Marakis (1988) probably because they did not take into acount the hyphal diameter, a factor that can alter the hyphal mechanical resistance (rigidity). It is known that the cell wall of the most fungi

is built up of interwoven microfibrils of chitin cemented by an amorphous matrix material. This structure affords ■ hyphal rigidity. So, when the skelletal component of the hyphae is reduced, the hyphal rigidity may become weaker resulting in an easier hyphal breakage during agitation of the culture (Gaitis & Marakis, 1994).

CONCLUSION

The reduction of the time needed for spore germination of *A. carbonarius* in the presence of Tween 80, is a very important result from a practical point of view, since it might contribute to the confrontation of problems (eg. production cost) due to a long lag phase of fungal spores used for the production of a fungal product with biotechnological processes.

The effect of Tween 80 on the germination time of fungal spores, should alert those scientists who are concerned with fungal growth for continuing the use of this surfactant as a soaking agent for the production of inoculum of spores since, even in very low amounts, it reduces the lag phase and generally affects the fungal growth (mycelium production and morphology, protein and RNA content etc.).

The presence of Tween 80 seriously affected the mycelium morphology, which is of considerable importance to the overall physicochemical environment within the fermenter, affecting this way the quality and production cost of a microbial product. So, the desirable mycelial form could be defined by addition or not of a proper amount of Tween 80 in culture medium.

ACKNOWLEDGEMENTS: The author would like to thank Dr G. Ouzounidou for encouragement and suggestions in preparing the manuscript.

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